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The conduction pore of a cardiac potassium channel

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Ion channels form transmembrane water-filled pores that allow ions to cross membranes in a rapid and selective fashion. The amino acid residues that line these pores have been sought to reveal the mechanisms of ion conduction and selectivity^{1–7}. The pore (P) loop⁸ is a stretch of residues that influences single channel-current amplitude, selectivity among ions and open channel blockade^{2,3,5} and is conserved in potassium-channel subunits previously recognized to contribute to pore formation^{5,9}. To date, potassium-channel pores have been shown to form by symmetrical alignment of four P loops around a central conduction pathway^{10–12}. Here we show that the selectivity-determining pore region of the voltage-gated potassium channel of human heart through which the I_{Ks} current passes includes the transmembrane segment of the non-P-loop protein minK. Two adjacent residues in this segment of minK are exposed in the pore on either side of a short barrier that restricts the movement of sodium, cadmium and zinc ions across the membrane. Thus, potassium-selective pores are not restricted to P loops or a strict P-loop geometry.

In the human heart, two voltage-dependent potassium channels, carrying the currents I_{Ks} and I_{Kr} , open during muscle contraction to repolarize the myocardium and end each heartbeat¹³. I_{Ks} channels are formed by assembly of KvLQT1, a subunit with a single P loop, with minK, a 129-residue protein with a single transmembrane segment^{14,15}. HERG, the P-loop subunit that mediates I_{Kr} , also coassembles with minK¹⁶. Controversy has surrounded the role of minK in these channels—some researchers argue that minK is a structural protein intrinsic to I_{Ks} -channel formation whereas others hold that it is an accessory subunit that functions as a channel regulator^{15–22}.

To determine whether minK sites are exposed in the I_{Ks} pore we performed scanning susceptibility analysis using the thiol-reactive transition-metal Cd^{2+} and 35 minK mutants, in each of which a single residue was mutated to cysteine in sequential positions along the transmembrane segment^{6,20,23}. In the preliminary screen, we evaluated channels composed of rat minK and a subunit native to *Xenopus* oocytes^{14,19,21,24}. Wild-type minK (Fig. 1) and a mutant in which the single native cysteine in minK was altered to alanine (C107A, not shown) were both insensitive to Cd^{2+} , whether the reagent was applied extracellularly or microinjected into the cytosol.

In contrast, serial mutation of residues 42–76 to cysteine produced two channels that were sensitive to external Cd^{2+} but remained insensitive to internal reagent; these channels carried F55C and G56C mutations (Fig. 1a, b). Two other channels, with F57C or T59C mutations, were sensitive to internal but not external reagent (Fig. 1a, b). Apparently, Cd^{2+} applied from the external side of the membrane could not move past residue G56 to reach position 57. Conversely, Cd^{2+} applied internally could interact with residue 57 but was unable to traverse the pore to reach position 56.

This pattern of reactivity was also observed for I_{Ks} channels formed with human minK (h-minK) when equivalent residues were mutated to cysteine. Both I_{Ks} channels (formed with wild-type h-minK and KvLQT1) and channels formed with KvLQT1 alone were insensitive to Cd^{2+} , whether applied from the external or internal solution (Fig. 2a, b). However, channels formed with F54C or G55C mutant h-minK were sensitive to external but not internal Cd^{2+} , whereas those with mutation F56C were sensitive to internal but not external reagent (Fig. 2a, b). The theory that there is a barrier to Cd^{2+} movement between residues G55 and F56 in the human I_{Ks} -channel pore is tenable only if Cd^{2+} blocks such movement by binding in and occluding the I_{Ks} -conduction pathway.

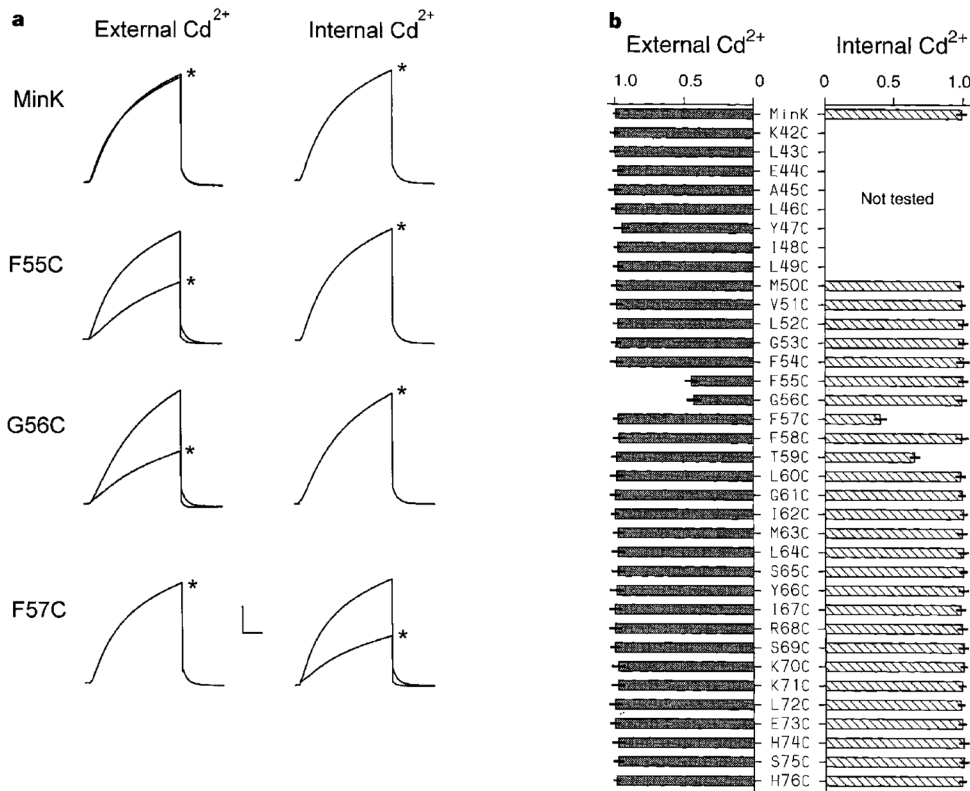


Figure 1 Blockade of channels formed with rat minK by Cd^{2+} . Whole-oocyte currents elicited by 10-s pulses from a holding potential of -80mV to 0mV with an interpulse interval of 20 s recorded by two-electrode voltage clamp are shown after leak subtraction. **a**, Current traces for sample oocytes before and after (asterisk) exposure to external Cd^{2+} (bath application of 0.5 mM CdCl_2 for 5 min) or internal Cd^{2+} (microinjection of 23 pmol CdCl_2 ; see Methods). Scale bars (bottom row) represent 200 nA (vertical bar) and 2 s (horizontal bar). **b**, The fraction of unblocked current at the end of a 10 s test pulse as described above for each mutant studied (mean \pm s.e.m. for 5–20 oocytes). cRNAs encoding minKs with cysteine residues at positions 62, 63, 65, 66, 69, 70 and 71 were co-injected with KvLQT1 cRNA (see Methods). Calculated inhibition constants were $0.035 \pm 0.003\text{ mM}$ for F55C, $0.11 \pm 0.01\text{ mM}$ for G56C and roughly 0.1 and 0.2 mM for F57C and T59C, as estimated previously²⁰. Internal Cd^{2+} produces a voltage-independent open state and decreased time-dependent currents through T59C channels (not shown).

Four characteristics of the inhibition by external Cd^{2+} indicate that it acts by a pore-blocking mechanism. First, blockade of G56C minK channels by external Cd^{2+} was voltage-dependent (Fig. 3a), as expected for a charged blocker that moves into the transmembrane electric field¹⁷. The block was rapidly reversible at this site and exhibited a δ value of 0.20 ± 0.03 ($n = 5$ oocytes), where δ is the fraction of the applied voltage drop experienced at the binding site. Second, block by external Cd^{2+} was sensitive to levels of permeant ions on the inside of the membrane (Fig. 3b). Raising intracellular levels of monovalent cations revealed that potassium or rubidium decreased the binding affinity of external Cd^{2+} at position G56C, whereas caesium had only a small effect and lithium was ineffective. Each cation decreased the affinity of external Cd^{2+} for the pore according to its position in the relative permeability series for open minK channels¹⁷, consistent with the notion that the internal test cations enter and traverse the conduction pore to destabilize Cd^{2+} on its external site^{20,25}.

Third, the kinetics of inhibition by Cd^{2+} in the absence and presence of external tetraethylammonium (TEA) also support a pore-blocking mechanism (Fig. 3c). External TEA is a weak but well characterized blocker of the I_{Ks} pore^{17,19,20}. As external TEA concentration was increased, the time constant (τ) of blockage by Cd^{2+} increased in a linear fashion directly proportional to the fraction of channels that were not blocked by TEA at equilibrium (Fig. 3d). This is expected for competitive inhibition by agents that reversibly block channels at overlapping binding sites²⁰. Finally, mutation of position 56 altered the ion-selectivity characteristics of the I_{Ks} channel. Mutant channels became permeable to Na^+ ions; this was revealed by inward sodium currents that were absent in wild-type channels (Fig. 3e). This change in selectivity was associated with a greater than five-fold increase in relative Na^+ permeability, as assessed by reversal potential measurements^{17,21}.

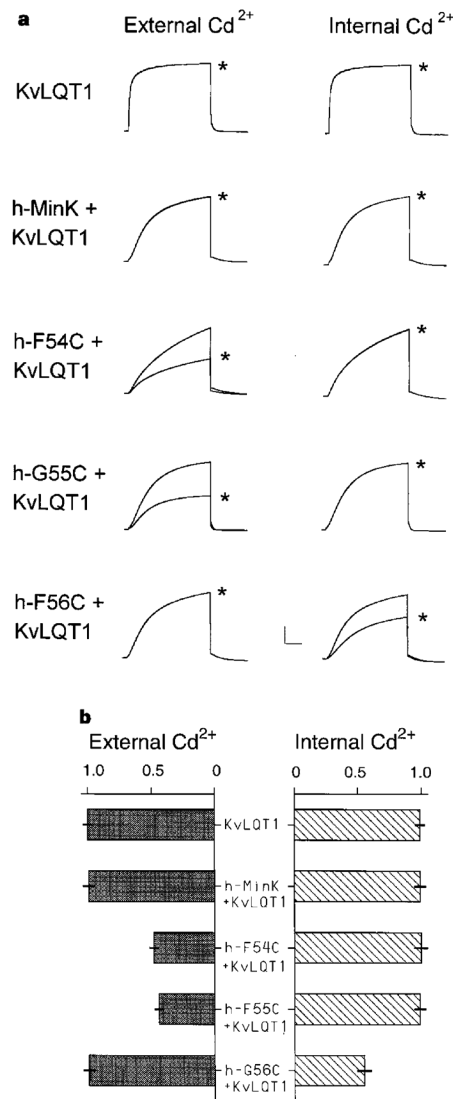


Figure 2 Blockade by Cd^{2+} of channels formed with KvLQT1 alone or with human minK and KvLQT1. **a**, Current traces for sample oocytes before and after (asterisk) exposure to external Cd^{2+} or internal Cd^{2+} , as in Fig.1. scale bars represent 200 nA (vertical bar) and 2 s (horizontal bar). **b**, The fraction of unblocked current (mean \pm s.e.m. for four to six oocytes) as in Fig.1. Calculated inhibition constants were $0.11 \pm 0.05 \text{ mM}$ for h-F54C + KvLQT1, $0.32 \pm 0.02 \text{ mM}$ for h-G55C + KvLQT1 and $\sim 0.3 \text{ mM}$ for h-F56C + KvLQT1, as estimated previously²⁰.

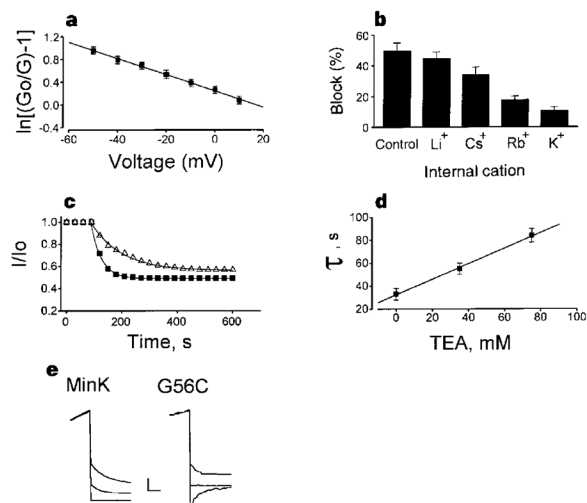


Figure 3 Inhibition of G56C rat minK channels by external Cd^{2+} shows the hallmarks of pore blockade. **a**, Blockage of G56C minK channels by external Cd^{2+} is voltage-sensitive; channels were opened by a 10-s command pulse to +20mV and current was measured 25 ms after repolarization to test potentials between -50 and +10mV; G0 and G, macroscopic conductances in the presence and absence of 0.5 mM Cd^{2+} , respectively (mean \pm s.e.m. for four oocytes). **b**, Block of G56C channels by external Cd^{2+} is altered by internal permeant cations; the fraction of blocked current by 0.5 mM external Cd^{2+} is shown before and after oocytes were injected with 20 nmol of the indicated salt solution; whole-cell currents were measured as in Fig. 1. **c**, External TEA slows the rate of block of G56C channels by external Cd^{2+} . The current was measured as in Fig.1, first with constant flow of ND-96 (see Methods) and then during solution exchanges to either 0.5 mM Cd^{2+} (solid squares) or 75 mM TEA solution followed by 0.5 mM Cd^{2+} with 75 mM TEA (open triangles). The curves are single-exponential fits to the Cd^{2+} -inhibition time course. **d**, The effect of TEA on the time constant (τ) for Cd^{2+} - mediated inhibition. The time constant for block in the absence of TEA is 39 ± 3 s. **e**, G56C minK channels are permeable to Na^+ ions whereas wild-type channels are not. Tail current reversal potentials (V_{rev}) were determined by opening channels with a 10-s pulse to +20mV and measuring current 50ms after shifting to test potentials from -70 to -150 mV in 40-mV steps; scale bars represent 200 nA (vertical bar) and 25ms (horizontal bar). Permeability ratios were calculated according to $P_K/P_{\text{Na}} = \exp(-FV_{\text{rev}}/RT)$ where R , T and F have their usual values and P_K and P_{Na} are measures of the permeability of K^+ and Na^+ ions, respectively. Wild-type V_{rev} is -150 ± 5 and 1 ± 1 in 98mM NaCl and KCl solutions, respectively, whereas G56C V_{rev} is -107 ± 5 and 1 ± 1 , giving a permeability ratio P_K/P_{Na} for wild-type channels of >350 and of ~ 50 for G56C channels. Values are mean \pm s.e.m. for 5–7 oocytes.

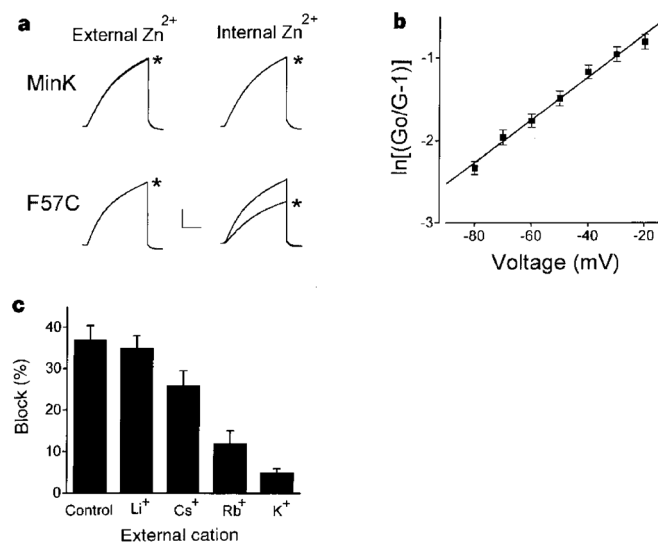


Figure 4 Inhibition of F57C rat minK channels by internal Zn^{2+} shows the hallmarks of pore blockade. **a**, Current traces for sample oocytes before and after (asterisk) exposure to external Zn^{2+} (bath application of 0.5 mM ZnCl_2 for 5 min) or internal Zn^{2+} (microinjection of 23 pmol ZnCl_2 ; see Methods) as in Fig. 1; scale bars represent 100 nA (vertical bar) and 3 s (horizontal bar). **b**, Block of F57C channels by internal Zn^{2+} is voltage-sensitive (protocol as in Fig. 3a; mean \pm s.e.m. for four oocytes). **c**, Block of F57C channels by internal Zn^{2+} is altered by external permeant cations; bars show the fraction of blocked current when Zn^{2+} -injected oocytes were studied in bath solutions containing 100 mM of the indicated chloride salt; currents measured as in Fig. 1 (mean \pm s.e.m. for four oocytes).

As the release of bound Cd^{2+} from the inside of F57C channels proceeds slowly (not shown), its use as a probe was limited. Zn^{2+} , however, blocked F57C channels from the intracellular but not external solution, did not block wild-type channels, and acted in a readily reversible fashion (Fig. 4a). As expected for an agent that acts by a pore-occlusion mechanism, blockade of F57C channels by internal Zn^{2+} exhibited both voltage dependence ($\delta = 0.33 \pm 0.06$ from the inside; $n = 6$ oocytes; Fig. 4b) and sensitivity to external monovalent cations according to the known permeability of the channel (Fig. 4c). Cysteine substitution of minK at positions 56 and 57 creates binding sites in the pore for external Cd^{2+} and internal Zn^{2+} , respectively, and neither metal can move inwards past G56C or outwards past F57C. This identifies these minK sites as boundaries of a selectivity barrier in the deep I_{Ks} pore.

The idea that a short region in the I_{Ks} pore selects against passage of Na^+ , Cd^{2+} and Zn^{2+} ions corresponds with the hypothesis that efficient movement of charges across cell membranes involves the limitation of unfavourable interactions of charges and low dielectric regions by close apposition of the bulk

solutions on either side of the membrane across a short restrictive pathway^{26–28}. The conduction pathways of *Shaker*^{6,7} and Kv2.1 (ref. 29) potassium channels may also have short barriers, as judged by the residues that are exposed in these pathways. Although the physical barrier to permeation in minK appears to be short, as much as half the transmembrane electric field is traversed between minK residues 56 and 57. Thus, external Cd²⁺ moves ~20% into the field to bind at position 56 (Fig. 3) whereas internal Zn²⁺ moves outward through ~30% of the field to position 57 (Fig. 4). These data do not differentiate between a molecular-sieve or a binding-site mechanism for selective permeation^{26,30}. Although the atomic diameter of impermeant Cd²⁺ is ~1.41 Å and that of K⁺ is ~2.66 Å, Cd²⁺ will be more heavily hydrated.

The transmembrane segment of minK has previously been detected in the external I_{Ks} pore²⁰. Here we show that this segment lines the deep pore. We are now working to establish the role of this segment in the formation of the cytoplasmic portion of the I_{Ks} conduction pathway and have succeeded in identifying minK sites that mediate blockade by internal TEA (K.K.T., F. Sesti and S.A.N.G., unpublished observations). These findings refute the theory that minK is simply a channel regulator and that its transmembrane segment is an inert membrane tether²². The data do not indicate the subunit stoichiometry of complete I_{Ks} channels—an intriguing issue given evidence for two minK monomers in complexes formed with the subunit endogenous to *Xenopus* oocytes¹⁹ and the likelihood that channels composed of KvLQT1 subunits in the absence of minK are tetrameric. The results also do not indicate whether P loops maintain a single conformation in different pores or modify their structure or function. That minK can co-assemble with P-loop subunits of at least two subfamilies (KvLQT1 and HERG¹⁶) argues such heteromultimeric pores will be found in other channels and reveals another mechanism for generation of potassium-channel diversity.

Methods

Molecular biology. Mutants of rat and human minK were made and sequenced as previously described^{20,21}. All cysteine mutants of rat minK were otherwise cysteine-free (C107A). Human minK and human KvLQT1 cDNAs were gifts from R. Swanson (Merck), and M. Keating and M. Sanguinetti (Utah)¹⁴, respectively.

Complementary RNAs were transcribed from rat minK genes in pSD^{20,21} and human minK and KvLQT1 in pBF2 (ref. 12). Two or more days after injection of 2 ng rat minK cRNA or 1 ng human minK cRNA and/or 5 ng human KvLQT1 cRNA, whole-cell currents were measured by two electrode voltage clamp under constant perfusion at 22 °C¹⁹. Data were filtered at 100 Hz and sampled at 1 kHz, and leak correction was performed off-line¹⁷.

Electrophysiology. Bath solution was either ND-96, as follows (in mM): 96 NaCl, 2 KCl, 1 MgCl₂, 0.3 CaCl₂ and 5 HEPES at pH 7.6, or solutions in which NaCl and/or KCl was replaced isotonicly by RbCl, CsCl, LiCl, CdCl₂, ZnCl₂ or TEACl. To study the effects of intracellular blockers, oocytes were microinjected with 23 nl unbuffered aqueous solutions containing 23 pmol ZnCl₂ or CdCl₂, 2.3 nmol TEACl or 20 nmol of the chloride salt of monovalent cations, as described²⁰, and were studied 30–60 min thereafter.

Recorded currents showed no evidence that I_{Ks} channels contained the native oocyte subunit two days after minK and KvLQT1 cRNAs were coinjected: channels formed by injecting only minK cRNA were blocked when the native subunit was modified by methanethiosulfonate (MTS)-ethylammonium or MTS-ethyltrimethylammonium²¹, whereas those containing minK and KvLQT1 were MTS-insensitive (not shown). When minK and KvLQT1 were coexpressed, ~60% of the current could be blocked one day after cRNA injection; by day 2 and thereafter, all channels were insensitive to MTS reagents, indicating replacement of sensitive native subunits by KvLQT1.

Of 35 positions mutated to cysteine in rat minK, 7 (I62C, M63C, S65C, Y66C, S69C, K70C and K71C) did not express current when complexed with the native *Xenopus* oocyte subunit. These mutants could, however, co-assemble with KvLQT1 and gain current expression at the cell surface. Coexpression of each of the seven mutants with KvLQT1 altered the fast, saturating activation kinetics seen in channels formed by KvLQT1 alone to the slow, non-saturating kinetics characteristic of I_{Ks} channels¹⁴.

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